Use of human-derived stem cells to create a novel, in vitro model designed to explore *FMR1* CGG repeat instability amongst female premutation carriers

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Received: 13 December 2017 / Accepted: 8 June 2018 / Published online: 20 June 2018
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Abstract

Objective Create a model, using reprogrammed cells, to provide a platform to identify the mechanisms of CGG repeat instability amongst female fragile X mental retardation 1 gene (*FMR1*) premutation (PM) carriers.

Methods Female PM carriers (with and without POI) and healthy controls were enrolled from June 2013 to April 2014. Patient-derived fibroblasts (FB) were reprogrammed to induced pluripotent stem cells (iPSC) using viral vectors, encoding KLF4, OCT4, SOX2, and MYC. *FMR1* CGG repeat-primed PCR was used to assess the triplet repeat structure of the *FMR1* gene. *FMR1* promoter methylation (%) was determined using *FMR1* methylation PCR (mPCR). Quantification of *FMR1* transcripts by RT-qPCR was used to evaluate the effect of reprogramming on gene transcription, as well as to correlate patient phenotype with *FMR1* expression. Production of *FMR1* protein (FMRP) was determined using a liquid bead array-based immunoassay.

Results Upon induction to pluripotency, all control clones exhibited maintenance of progenitor cell CGG repeat number, whereas 10 of 12 clones derived from PM carriers maintained their input CGG repeat number, one of which expanded and one contracted. As compared to parent FB, iPSC clones exhibited a skewed methylation pattern; however, downstream transcription and translation appeared unaffected. Further, the PM carriers, regardless of phenotype, exhibited similar *FMR1* transcription and translation to the controls.

Conclusions This is the first study to establish a stem cell model aimed to understand *FMR1* CGG repeat instability amongst female PM carriers. Our preliminary data indicate that CGG repeat number, transcription, and translation are conserved upon induction to pluripotency.

Keywords iPSC · Stem cell · *FMR1* · Premutation · Primary ovarian insufficiency · CGG repeat

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Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10815-018-1237-y>) contains supplementary material, which is available to authorized users.

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Background

Although the majority of causes of primary ovarian insufficiency (POI) remain unknown, it is well established that the single gene that is most significantly associated with primary ovarian insufficiency (POI) is the fragile X mental retardation 1 gene (*FMR1*) at Xq27.3 [1]. Caused by expansion of cytosine-guanine-guanine (CGG) repeats at the 5' untranslated region of the *FMR1* gene, inheritance of greater than 200 repeats leads to fragile X syndrome (FXS), the most common cause of inherited intellectual disability. The number of cytosine-guanine-guanine (CGG) repeats in the fragile X mental retardation (*FMR1*) gene has commonly been defined as normal (< 45), intermediate (45–54), premutation (PM) (55–200), and full mutation (> 200) [2].

PM alleles (55–200 CGG repeats) are relatively common in the general population occurring in 1 in 130–250 women and in 1 in 250–810 men [3]. With a peak risk for fragile X-associated POI (FXPOI) between 70 and 100 CGG repeats [4, 5], 16–24% of PM carriers will experience primary ovarian insufficiency in some severity [6, 7], as compared to 1% of the general population [8, 9]. Moreover, despite higher prevalence of impaired or even abolished ovarian reserve, it is important to note that women with FXPOI can still become pregnant and may have a child with fragile X syndrome [10]. Although the current accepted understanding of CGG expansion is that of occurring during female meiosis, the details and timing of *FMR1* CGG instability have yet to be fully elucidated.

To date, we know very little about the mechanisms leading to FXPOI. Because of hypermethylation and subsequent gene silencing, it is established that full mutation carriers exhibit normal ovarian function [5]. Thus, a potential reduction in *FMR1* protein (FMRP), in and of itself, does not appear to be the underlying problem. With varying levels of *FMR1* promoter methylation, PM carriers, however, maintain gene transcription. In fact, because of the increased numbers of CGG repeats (most distinctly amongst mid-range PM > 80 CGG repeats), PM carriers tend to exhibit elevations of *FMR1* gene transcripts (1.5- to 4-fold amongst female PM carriers), which have been suggested to have a “gain of toxicity” effect at the level of the ovaries, as is the case for the other PM-associated disorder, fragile X-associated tremor/ataxia syndrome (FXTAS) [1, 11–13]. Importantly, however, this hypothesis has not yet been tested in human patients with advanced FXPOI.

Because of limited access to human germ cells and ovarian tissue of reproductive aged women, in particular those with PM and FXPOI, our ability to truly understand the mechanism leading to FXPOI and CGG expansion of the maternally inherited PM has been limited. In addition to extrapolation from pathophysiological findings in FXTAS, existing murine model systems designed to recapitulate FXPOI have been informative. Investigation of wild-type (WT) animals has revealed FMRP expression in granulosa cells (GC), luteal cells (LC), and oocytes at all follicle stages [14–17]. In fact, one model reported higher

concentration of FMRP in ovarian tissue, as compared to the brain [14]. Additionally, *FMR1* mRNA expression was confirmed in GCs, primordial, pre-antral, and early antral follicles, with a decrease in *FMR1* transcription in preovulatory follicles [15, 16]. Amongst those with a genetically introduced PM, ranging from 90 to > 130 CGG repeats, as compared to WT animals, researchers consistently reported an increase in transcription of *FMR1* mRNA within the granulosa cells and oocytes [14, 15, 17]. Upon characterization of the ovarian tissue, the mice with PM exhibited smaller ovaries and impaired follicular development and recruitment, GC abnormalities, and luteinizing hormone (LH) receptor downregulation as compared to WT [14, 15]. Despite this extensive effort, work in primate and human models is still needed.

The use of stem cells to model disease systems allows researchers to use patient-derived induced pluripotent cells (iPSCs) to (i) potentially recapitulate the step-by-step development of disease, (ii) discover the underlying molecular mechanisms involved in the disease pathology, and (iii) apply existing and emerging approaches for discovering and testing different classes of therapeutics that target early steps in disease pathogenesis [18]. Despite the ability to successfully differentiate iPSCs into varying cell types of the three embryonic germ cell layers (endoderm, mesoderm, ectoderm), researchers are still working to define an efficient, reliable method to derive patient-specific primordial germ cells in vitro. With promising work on in vivo derivation of human male primordial germ cells (PGCs) [19, 20], a recent study by Leng et al. was the first to report production of PGCs derived from two POI patients with X chromosome abnormalities [21]. With the ability to reliably produce patient-derived PGCs that with proper conditioning mature to meiotically competent oocytes on the near horizon, there is promise that researchers may be able to identify patient-specific repeat length instability, cellular and signaling defects, and cell viability in in vitro-derived GCs and PGCs. Further, this work could provide a platform to explore novel defects in somatic cells that support follicle survival and maturation that may contribute to POI, such as mitochondrial dysfunction [5, 22].

Objective With an overall objective to understand the unique pattern of *FMR1* CGG repeat instability amongst meiotically active oocytes, the goal of this study is to create a model, using reprogrammed cells, to provide a platform to identify the mechanisms of repeat instability and ovarian dysfunction amongst female premutation carriers.

Experimental procedures

Clinical characterization

Upon enrollment, all subjects completed a standard intake form, reporting their full medical history, with particular

emphasis on reproductive and endocrinologic history. To characterize their ovarian reserve, all subjects underwent a pelvic ultrasound, measuring the uterine volume, endometrial thickness, bilateral ovarian volumes, and antral follicle count (AFC) where each follicle from 2 to 10 mm was included, as well as documentation of any ovarian cysts. All subjects had a minimum of one hormone-free month, prior to undergoing serum assessment of ovarian reserve. All patients had the following studies: estradiol, follicle stimulation hormone (FSH), luteinizing hormone (LH), inhibin B, and antimüllerian hormone (AMH). Those that were still regularly menstruating underwent venipuncture during the early follicular phase (cycle days 2–5), and those with amenorrhea underwent sampling on the day of their exam. A single reference laboratory analyzed all samples.

Cell culture

Fibroblasts were isolated from a 4-mm punch biopsy of each subject. They were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-Invitrogen) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Once confluent, the cells were passaged using 0.05% trypsin. Each line was passaged four to five times prior to reprogramming. Cell pellets were obtained for CGG repeat length, CpG island methylation, *FMR1* expression, and fragile X mental retardation protein (FMRP) quantification.

iPSC generation

Fibroblasts were cultured up to a passage of 5 (as above) in a 6-well plate in fibroblast medium up to 50–80% confluence on the day of transduction. The cells were then transduced using the CytoTune® 2.0 Sendai reprogramming vectors, encoding *KLF4*, *OCT4*, *SOX2*, and *MYC*. After an overnight incubation, the reprogramming vectors were removed with medium replacement of fresh complete fibroblast medium (DMEM containing 10% FBS, 1% non-essential amino acids solution (10 mM), and 0.1% β -mercaptoethanol (55 mM)). One week post-transduction, the cells were passaged using 0.05% trypsin/EDTA and plated on γ -irradiated mouse embryonic feeder (MEF) cells until the iPSC colonies could be picked. Medium was transitioned from fibroblast to iPSC medium (KnockOut™ DMEM/F12 with 20% KnockOut™ serum replacement, 1% MEM Non-Essential Amino Acids Solution, 10 mM, 1% GlutaMAX™-I, 0.1% β -mercaptoethanol (55 mM), 1% penicillin-streptomycin, and 0.04% bFGF (10 ng/mL)), the day following passage to MEF colonies. In addition to morphology, live staining with Tra1-81 aided colony selection for expansion. Three distinct clones from each subject were selected and cultured in iPSC medium, using MEF feeders for two passages. At the third passage, and thereafter, the clones were cultured in mTeSR1

on matrigel-coated plates (feeder-free). Each colony was passaged every 4 to 7 days with 0.02% EDTA up to eight passages. These cells were used to assess *FMR1* CGG repeat length, affirm methylation status of the *FMR1* promoter, and quantify mRNA transcription and FMRP levels.

Teratoma formation

iPSC clones were plated onto matrigel prior to transplantation, reaching approximately 80% confluence. Cells were then harvested by scraping and resuspended in 30 μ L of matrigel (1:1 dilution in PBS). For each clone, two nude-SCID gamma (NSG) mice were anesthetized according to the approved animal protocol (IRB Aplac 21,636). A small incision was made along the spine, approximately 0.5 cm below the lowest rib. The kidney was then located visually, and an incision was made into the peritoneal cavity. The kidney was then brought to the incision, a small hole in the capsule membrane created with sharp dissection, and 30–50% of the cells were injected beneath the kidney capsule using a transfer pipette. The kidney was then replaced, and the peritoneal cavity closed with dissolvable suture and the skin with wound clips. The remaining volume of cells was then injected subcutaneously. Thereafter, mice were monitored every other day for tumor growth. Four to 6 weeks after transplantation, the mice were sacrificed and processed according to approved protocols. Teratomas were harvested and fixed in paraffin for hematoxylin and eosin staining.

Karyotype analysis

Sample (pelleted from iPSC clones) and control DNA were labeled with Cy3 and Cy5 fluorophores following the manufacturer's instructions. Labeling mixes were combined and hybridized on 24sure arrays™ (v3, BlueGnome, Cambridge, UK) for 6–12 h. Fluorescence intensity was detected using a laser scanner (Powerscanner, TECAN, Mannedorf, Switzerland), and BlueFuse Multi 2.6 software was used for data processing (BlueGnome, Cambridge, UK).

Immunocytochemistry

Live staining was used to identify reprogrammed colonies, prior to clonal expansion. Cells were washed once with KnockOut™ DMEM/F12. The diluted primary antibody (Tra-1-81, 1:100, Stemgent) was then applied, and the cells were incubated at 37 °C for 60 min. The primary antibody was then aspirated, and the cells washed with KnockOut™ DMEM/F12. The cells were then incubated in the diluted secondary antibody (Invitrogen) for 60 min at 37 °C. The secondary antibody was aspirated, the cells washed again with KnockOut™ DMEM/F12, and visualized under a standard fluorescent microscope.

Induced pluripotent stem cells were washed once with PBS, fixed with 10% formalin solution (Sigma) for 20 min, and then washed again with PBS. Cells were permeabilized with 0.05% Triton X-100 for 5 min (only for NANOG and OCT4) and then incubated for 1 h in the blocking buffer (10% bovine serum albumin (BSA)). Primary antibodies were applied, diluted in the blocking solution, and incubated overnight incubation at 4 °C. Cells were then washed and treated with the appropriate fluorophore-conjugated secondary antibody (Invitrogen). After a 1-h incubation at room temperature, the cells were washed. For nuclear visualization, a 2 µg/mL working solution of DAPI (4',6-diamidino-2-phenylindole) was applied, incubating for 10 min at room temperature. The cells were once again washed and mounted with mounting medium for imaging. The antibodies used for pluripotency staining were mouse anti-human Tra-1-60 and SSEA4, and rabbit antihuman NANOG (1:100 Stemgent).

CGG repeat length and AGG interruption analysis

Genomic DNA was precipitated from pelleted cells using a silica-based DNA purification spin column (DNeasy Blood & Tissue Kit, Qiagen). DNA quantification was performed using the NanoDrop 2000 spectrophotometer. Samples were diluted in 10 mmol/L Tris and 0.5 mmol/L EDTA, pH 8.8, to 20 ng/µL prior to PCR.

PCR was then performed to assess the number of CGG repeats in the 5' untranslated region of the *FMR1* gene. Samples were PCR-amplified using AmpliX® PCR/CE *FMR1* reagents (no. 49402) according to prior reports [23]. Specifically, a master mix containing 11.45 µL of GC-rich AMP buffer (no. 145152), 0.5 µL of FAM-labeled *FMR1* primers (no. 145151), 0.5 µL of *FMR1* CGG primer (no. 145156), 0.5 µL of nuclease-free water, and 0.05 µL of GC-rich polymerase mix (no. 145153) from Asuragen, Inc. (Austin, TX) was used to amplify the DNA. The gene-specific primer sequences were published previously [24], and the CGG-specific primer followed the design of the sequence provided by Tassone et al. [25] with a (CGG)₅ anchor sequence. Reagents were vortexed before dispensing to a microtiter plate (96-well plates, Phenix Research Products, Chandler, NC). Aliquots of the DNA sample, typically 2 µL at 10 ng/µL, were transferred to the plate. Sealed plates (Aluminum film sheets, Phenix Research Products) were vortexed, centrifuged, and transferred to a thermal cycler (9700, Applied Biosystems, Foster City, CA). Samples were amplified with an initial heat denaturation step of 95 °C for 5 min, followed by 10 cycles of 97 °C for 35 s, 62 °C for 35 s, and 68 °C for 4 min, and then 20 cycles of 97 °C for 35 s, 62 °C for 35 s, and 68 °C for 4 min with a 20 s autoextension at each cycle. The final extension step was 72 °C for 10 min. Upon completion of PCR, samples were immediately analyzed by capillary electrophoresis.

All amplicons were analyzed on an ABI 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA) as described [24]. A total of 2 µL of each PCR product was mixed with 2 µL of ROX 1000 size ladder (no. 145154) and 11 µL of HiDi Formamide (Applied Biosystems), and 2 µL of the mixture used for injection. Samples were heat-denatured at 95 °C for 2 min followed by cooling on ice prior to transfer to the CE instrument. Data analysis of PCR products detected by CE was analyzed using GeneMapper 4.1 software (Applied Biosystems) consistent with previous descriptions [24].

Identification of AGG interruptions in each sample was achieved using the methods described in Chen et al. [21].

FMR1 promoter methylation

FMR1 promoter methylation was assessed using the AmpliX® mPCR *FMR1* kit (no. 49442, Asuragen, Inc., Austin, TX) in accordance with the manufacturer's protocol. In short, two aliquots of 40 ng gDNA (4 µL each of 10 ng/µL) from each sample were prepared for methylation assessment using mPCR reagents. One aliquot was mixed with 4 µL digestion enzyme mix (containing HpaII) and the other with 4 µL digestion control mix (containing digestion buffers). Upon completion of digestion, separate PCR master mixes of methylation PCR reagents were added to the respective reactions. Each master mix contained mPCR primers that flanked the repeat element, and two HpaII sites as previously described by Chen et al. [26], with the exception that the FAM and HEX-labeled primers, were added to the control and HpaII-digested reactions, respectively. PCR was performed per prior published protocol [26]. Upon completion of PCR, samples were immediately analyzed by CE (ABI 3500xL Genetic Analyzer, Applied Biosystems) and separated using POP7 polymer (Applied Biosystems) following the manufacturer's instructions. PCR products detected by CE were analyzed using GeneMapper 4.1 software (Applied Biosystems) as previously described [22].

FMR1 transcription analysis

RNA was precipitated from cell pellets according to the manufacturer's protocol (RNeasy Plus Mini Kit, Qiagen) and quantified using a NanoDrop2000 spectrophotometer. Synthesis of cDNA was performed in accordance with the manufacturer's protocol (Invitrogen). Next, 1 µL of the newly synthesized cDNA was used for quantitative PCR (qPCR) amplification, using the 7900HT Fast Real-Time PCR system (Applied Biosystems). Of the 24 predicted *FMR1* mRNA isoforms, primers were selected to detect total *FMR1* mRNA and isoforms 10 and 10b (see supplemental Fig. 1 for primer sequences). Added to a mixture of 2× QuantiFast SYBR® Green PCR Master Mix and RNase-Free water, 1 µM of forward primer and 1 µM of reverse primer, to a volume of

10 μL , the qPCR reaction was performed according to established protocol (Qiagen). The relative concentration of total *FMRI* mRNA and isoforms 10 and 10b, as well as variations encountered during sample preparation, was assessed by use of a reference amplicons, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and ubiquitin-specific peptidase 33 (*USP33*). A total of 40 cycles were performed, with the following temperature settings: 50 °C for 2 min, 95 °C for 10 min, and 95 °C for 15 s, followed by 60 °C for 1 min. Melting curves were used to determine primer accuracy. In addition to using enrolled patient controls, the following known assay controls were used: a normal female with 30/30 CGG repeats and a full mutation male > 200 CGG repeats. A negative control included analysis without the addition of cDNA. Performed in triplicate, quantification cycle (C_q) values were obtained using SDS2.3 (Applied Biosystems). The expression levels of transcripts were calculated by the relative quantification ($\Delta\Delta C_q$) study method from PCR replicates, where the C_q values obtained from enrolled control samples were pooled together. Data were then expressed as a fold change relative to the control group.

FMRP quantification

Cell pellets obtained from fibroblasts (FB) and unique iPSC colonies were washed in PBS and lysed in an extraction reagent (Pierce M-PER mammalian protein extraction reagent; Thermo Fisher Scientific, Rockford, IL) containing 150 mmol/L NaCl, 10 mg/mL chymostatin, 10 mg/mL aprotinin, and 1 $\times$ protease inhibitor cocktail (Complete mini tablets, EDTAfree; Roche Applied Science, Indianapolis, IN). After a brief sonication, cell debris was removed by centrifugation at 16,000g for 15 min; the protein concentration in the supernatant was determined using a Pierce BCA protein assay (Thermo Fisher Scientific). FMRP assays were performed with the anti-FMRP mouse monoclonal antibody mAb6B8 (MMS-5231, Covance Inc., Dedham, MA) and the anti-FMRP rabbit polyclonal antibody R477 [27]. These antibodies are highly specific and each recognizes a different epitope of the protein. A GST fusion protein, GST-SR7 carrying an abbreviated sequence of FMRP that includes the epitopes of mAb6B8 and R477, was used as standard [20]. The immunoassays were performed as previously described [5]. Briefly, 50 μL cell lysate eluate was incubated for 6 h with 3000 $\times$ MAP-MicroPlex microspheres (Luminex, Austin, TX) coupled to anti-FMRP mAb6B8. The microspheres were then washed and incubated overnight with rabbit antibody, R477 that was subsequently labeled for 2 h by phycoerythrin-conjugated goat anti-rabbit IgG. FMRP was quantified with a Luminex 200 system. Dilutions of GST-SR7 were used to generate an FMRP standard concentration curve for each 96-well plate analyzed. The amount of FMRP in the sample was reported as concentration (pM) in the 50 μL cell lysate eluate.

Statistics

Analysis between two groups was made using Student's *t* test for independent parametric values and paired *t* test for dependent variables (e.g., comparison of clonal expression of *FMRI* iso 1 and 10/10b). Qualitative variables were analyzed with Fisher's exact test. Statistics were performed with StataCorp (*Stata Statistical Software: Release 13*. College Station, TX: StataCorp LP). For statistical analyses, a $p < 0.05$ was considered to be statistically significant, although a Bonferroni correction for multiple testing was applied to transcription analysis, and $p < 0.05/3$ (3: number of clones analyzed) was considered significant.

Results

Patient demographics

Four PM carriers and three control subjects were enrolled and clinically characterized. All women were of reproductive age, ranging 26 to 39 years. Amongst the PM carriers, two women exhibited clinical and biochemical signs of primary ovarian insufficiency, with secondary amenorrhea and menopausal hormone levels (see Table 1). Additionally, two of the PM carriers have children. Specifically, patient 3 has a son with FXS, two children who are PM carriers, one is awaiting test results, and three who have CGG repeats within the normal range. Patient 2, who is the sister of patient 3, has a developmentally normal daughter with untested *FMRI* CGG repeat number, and desires more children, but exhibits evidence of POI. Patients 1, 2, and 4 were tested for *FMRI* repeat number during an assessment of infertility and ovulatory dysfunction. While patients 1 and 2 exhibit a more severe form of POI, patient 4 has diminished ovarian reserve, as compared to those of her same age, but still cycling monthly. Interestingly, all four women have PM within a narrow range of 78–83 CGG repeats on allele 2. The control subjects are similar to the study subjects, with regard to age, gravidity, and parity ($p = \text{NS}$). As compared to the control subjects, the PM carriers exhibit a difference in ovarian reserve parameters (AFC ($p = 0.05$), AMH ($p = 0.04$), FSH ($p = 0.12$)) and size of CGG repeat on *FMRI* allele 2 ($p < 0.00001$).

Fibroblast characterization

Fibroblasts from four PM carriers and three controls (both in CGG repeat number and ovarian function) were analyzed for *FMRI* CGG repeat number, methylation pattern, and *FMRI* expression. As anticipated, all control subjects exhibited a normal CGG repeat number (< 45; ranging 23–37). Furthermore, the repeat number within the cultured FB was consistent with CGG repeat sizing of serum leukocytes (± 1

Table 1 Baseline patient demographics

	Patients with FMR1 PM				Control subjects			<i>p</i> value
	1	2	3	4	5	6	7	
Age	28	33	39	32	26	32	32	0.19
Secondary amenorrhea	Yes	Yes	No	No	No	No	No	0.15
Gravidity	0	1	9	0	0	1	0	0.66
Parity	0	1	7	0	0	1	0	0.66
AFC	2	1	11	8	17	8	38	0.05
AMH	<0.1	0.10	1.37	1.13	2.47	1.26	5.56	0.04
FSH	49.10	86.30	6.42	11.30	8.92	7.34	8.45	0.12
FMR allele 1	30	32	32	23	30	30	23	0.32
FMR allele 2	82	78	83	78	37	33	30	<0.00001

Comparison of *FMR1* premutation carriers (PM) to reproductive-aged female controls were made using Fisher's exact test for all categorical variables and Student's *t* test continuous variables

AFC antral follicle count, *AMH* antimüllerian hormone, *FSH* follicle stimulating hormone

CGG repeat). The FB derived from the PM carriers exhibited similar stability (± 1 CGG repeat) to the serum leukocytes. Additionally, the AGG structure appeared to be preserved between leukocytes and FB for all samples. Using AmpliX® *FMR1* mPCR, we found that all PM carriers exhibited a phenomenon of size and methylation mosaicism, consistent with previous descriptions of heterogeneity in this range (see Table 2) [26]. Of interest, the DNA derived from leukocytes and FB exhibited a dissimilar pattern in methylation. Specifically, the DNA derived from fibroblasts, on average, had a lower percent methylation across both alleles than that of the leukocytes (31.8 vs. 52.8%, respectively; $p = 0.0008$).

FMR1 expression (total *FMR1* mRNA (isoform 1) and isoforms 10 and 10b) was assessed relative to *USP33* and *GAPDH* transcripts, both previously determined to be free of FMRP binding [28]. Of note, both reference genes exhibited correlated expression levels ($R^2 = 0.84$), and therefore herein, we will discuss isoform expression relative to *GAPDH*, for simplicity. The relative expression of total *FMR1* mRNA (isoform 1) to *GAPDH* differed amongst the PM carriers and controls ($p = 0.007$), where the PM carriers (collectively) expressed 40% less *FMR1* mRNA than the controls (see Fig. 2). However, the deviation from the controls was < 1 Cq unit. As compared to total *FMR1* mRNA, the relative transcript levels of isoforms 10 and 10b amongst both the PM carriers (patients 1–4) and controls were similar to those of isoform 1 (see Fig. 2), with a slightly higher relative production of isoform 10/10b (fold change of 0.77 vs 0.61, respectively ($p = 0.03$)). Comparing the total *FMR1* transcript patterns amongst the PM carriers, while controlling for *FMR1* transcription amongst the control subjects, the PM carriers exhibited a phenotype-associated difference in expression ($p = 0.004$). Patients 1, 2, and 4 exhibited relatively lower total *FMR1* (isoform 1) (fold change 0.63 ($p = 0.01$), 0.56 ($p = 0.006$), and 0.53 ($p = 0.006$), respectively) as compared to

controls, whereas expression patterns of patient 3 were similar to those of the controls (fold change 0.76, $p = 0.036$, where a p value of < 0.0125 was considered significant) (see Fig. 2c). This expression pattern was similar when we examined the relative expression of isoform 10/10b amongst the PM carriers (see Fig. 2d).

Lastly, FMRP production levels amongst the PM carriers (overall) and controls were similar (28.65 and 27.70 pg/μg lysate, respectively, $p = 0.81$). Comparing each individual PM to that of the controls suggests an increase in FMRP production amongst the PM carrier with normal ovarian reserve, relative to her FXPOI counterparts, and that of the controls (see Table 4).

Fibroblast reprogramming and iPSC characterization

Using the non-integrating Sendai reprogramming vectors, encoding *KLF4*, *OCT4*, *SOX2*, and *MYC*, the FB were successfully reprogrammed to iPSC with an efficiency of 0.2–4.4%. All iPSC clones demonstrated expected characteristics of pluripotent stem cells: morphology similar to embryonic stem cells (see Fig. 1a), immunoreactivity for NANOG, Tra1–60, Tra1–81, and SSEA4 (see Fig. 1b), reactivation of genes indicative of pluripotency (see Fig. 1c), and ability to form in vivo teratomas with differentiation into endoderm, mesoderm, and ectoderm (see Fig. 1d). Karyotypic analysis confirmed that the iPSC lines maintained a normal diploid karyotype (46, XX).

Effect of reprogramming on CGG repeat length and methylation patterns

As anticipated, upon induction to pluripotency, nine out of nine clones derived from control subjects maintained the CGG repeat number of their progenitor cell (FB). Amongst

Table 2 Analysis of *FMRI* CGG repeat number and promoter methylation

Patient	Cell type	Peak 1			Peak 2			Peak 3		
1	Leuk	30	54	5	83	44	2	85	2	0
	FB	30	37	2	82	24	1	84	16	2
	iPSC A1	30	98	4	82	1	0	..		
	iPSC A2	30	100	0	51	2	1			
	iPSC A3	30	100	0	82	1	0			
2	Leuk	32	39	1	78	84	6	81	4	2
	FB	32	23	1	78	46	4			
	iPSC A1	32	56	1	78	48	2			
	iPSC A2	32	6	0	78	99	2			
	iPSC A3	32	2	0	78	97	4			
3	Leuk	32	75	0	84	56	1	87	6	0
	FB	32	22	5	83	44	5			
	iPSC A1	32	69	3	83	1	0			
	iPSC A2	32	100	1	89	3	1			
	iPSC A3	32	100	0	83	3	0			
4	Leuk	23	68	3	78	24	4			
	FB	23	14	1	77	63	8			
	iPSC A1	23	9	1	77	100	0			
	iPSC A2	23	2	0	77	100	0			
	iPSC A3	23	2	0	77	100	0			
5	Leuk	30	61	6	37	51	2			
	FB	30	27	1	37	28	1			
	iPSC A1	30	2	1	37	92	12			
	iPSC A2	30	2	0	37	100	0			
	iPSC A3	30	100	0	37	2	0			
6	Leuk	30	47	1	33	49	4			
	FB	30	22	1	33	42	1			
	iPSC A1	30	3	1	33	100	0			
	iPSC A2	30	3	1	33	100	0			
	iPSC A3	30	100	1	33	5	1			
7	Leuk	23	30	1	30	68	1			
	FB	23	16	0	30	37	2			
	iPSC A1	23	98	3	30	2	0			
	iPSC A2	23	2	1	30	100	0			
	iPSC A3	23	98	3	30	2	1			

As the methylation assay was performed in duplicate, the relative percent of allele-specific methylation is reported as an average. Sizes 1–2 demonstrate CGG repeat size of alleles 1 and 2; size 3 (only seen in PM carriers) demonstrates mosaicism in CGG repeat size and methylation of the PM range allele. iPSC A1–3 indicate each iPSC colony

% Meth % methylation, *Leuk* leukocytes, *FB* fibroblasts

the PM carriers, 10 out of 12 clones maintained their progenitor cell CGG repeat number within a single repeat unit. Two subjects, patients 1 and 3, each had a single clone that exhibited instability. While the first, patient 1, clone A2, underwent contraction in CGG repeat number from 82 to 51, patient 3, clone A2, expanded from 83 to 89 repeats (see Table 2).

Indications of interrupting AGG sequences were maintained upon induction to pluripotency in all patients and controls. Interestingly, upon induction to pluripotency, the methylation pattern differed dramatically from that of both the leukocytes and FB. Across all clones, including iPSCs derived from control subjects, *FMRI* promoter methylation was highly skewed between alleles. Exhibiting evidence of a near lack of methylation (1–2%) to full methylation (98–100%), the alleles (PM versus normal CGG repeat number) did not appear to preferentially become hyper or hypomethylated, with variances within and between sample lines. Of note, the two clones that exhibited CGG instability revealed hypomethylation of the PM allele.

Effect of reprogramming on *FMRI* expression and FMRP production

Upon induction to pluripotency, the pattern of *FMRI* expression (both *FMRI* total (isoform 1) and isoforms 10 and 10b) from both the PM carriers and controls was overall similar to that of their parent FB. Transcription of *FMRI* (iso 1) amongst the iPSCs derived from the PM carriers (pooled) yielded a fold change of 0.57 relative to controls, as compared to 0.61 amongst the parent FB (pooled), ($p = 0.76$). Examination of iso 10/10b exhibited a nonsignificant elevation in transcription in the pluripotent state. Where amongst the parent FB expression of iso 10/10b was 0.78-fold relative to controls, the iPSCs relative expression was 1.08-fold ($p = 0.05$). Given that iPSC clones exhibited unique methylation patterns, clonal transcription patterns were also assessed. Independent of methylation pattern, the iPSC clones exhibited individual variances in *FMRI* (iso 1 and iso 10/10b) production (with deviation of less than 1 Cq unit) (see Table 2, Fig. 3 and supplemental Fig. 2).

Analysis of FMRP production exhibited a nonsignificant trend towards an overall increase in FMRP translation amongst FB, relative to iPSC. Comparing clonal translation patterns of FMRP amongst PM carriers, we found that patient 3 exhibited a nonsignificant increase in FMRP production, as compared to the PM carriers with POI (patients 1, 2, and 4), mirroring FMRP production in the input FB (see Tables 3 and 4, respectively).

Discussion

This is the first study to establish a human stem cell model for *FMRI* CGG repeat instability and POI amongst female PM carriers. Furthermore, this is the first study to explore *FMRI* DNA, RNA, and protein in an iPSC model system. Such a model system is of particular importance in understanding molecular mechanisms of both *FMRI* CGG repeat instability and that of the PM on human ovarian function, as non-invasive methods to discriminate such mechanisms are

Fig. 1 Establishing pluripotency of iPSCs. Emerging iPSC colonies demonstrating characteristic iPSC morphology (**a**). Colonies were selected for expansion after meeting morphologic criteria and live staining with Tra-1-81 (**b**). Expression patterns of iPSC colonies are consistent with pluripotency, where colonies A, B, C, and D are unique colonies derived from patients 1, 2, 3, and 4, respectively. The number next to the letter indicates primary antibody: 1 = NANOG, 2 = SSEA4,

and 3 = Tra-1-60 (**c**). Comparison of FB to iPSC derived from patients 4 and 5 demonstrates lack of production of SOX2 from the FB, where the star is the place holder for the relative expression of SOX2 in FB, whereas the iPSC exhibited a 2-fold increase in expression (**d**). Lastly, teratoma formation confirmed that the iPSCs were capable of differentiating into mesoderm (i), endoderm (ii), and ectoderm (iii) (**e**)

currently not available. Using the non-incorporating Sendai virus, our pluripotent stem cells exhibit characteristic markers of pluripotency. Importantly, upon induction to pluripotency, the following parameters were conserved: pattern of AGG interruptions, CGG repeat number (in nearly all cell lines), *FMR1* transcription, and FMRP production.

Of interest is the *FMR1* CGG repeat number upon induction to pluripotency. Whereas nine of nine clones derived from the control patients exhibited stability (± 1 CGG repeat) upon induction to pluripotency, as compared to the parent FB, two clones within the cohort of iPSC derived from the PM carriers demonstrated CGG instability. Specifically, clone A2 of patient 1 contracted from 82 CGG repeats to 51, and clone A2 of patient 3 expanded from 83 to 89 CGG repeats. These findings are particularly intriguing, as whether somatic expansion involves the same mechanisms as intergenerational expansion is unknown. Where intergenerational expansion is thought to be attributable to meiotic aberrations in DNA repair within the gamete [29], Lokanga et al. [30] have identified somatic expansion within PM knock-in mouse models as well as within a human lymphoblastoid cell line, GM06891, over a period of

42 months. Originally starting with ~ 118 repeats, in 2 years of intermittent propagation, samples isolated exhibited limited quantities of the original allele, and alleles that were ~ 16 , ~ 35 , and 51 repeats larger were apparent (of which the largest was the predominating allele). In combination with female PM carriers having children with varying CGG repeat numbers, the somatic expansion previously reported by Lokanga et al., and seen within our sample set, calls to question whether or not instability occurs during the mitotic expansion of gonocytes that occurs prior to oocyte entry into female meiosis. Alternatively, this instability may not necessarily be related to mechanisms that contribute to expansion *in vivo*, as the work here is several steps removed from any “physiologic” state. Certainly, with the ability establish meiotically competent germ cells in the near future, it will be of great interest to utilize this model to explore expansions that occur in the primordial germ cell state, granulosa cells, and oocytes.

As epigenetic regulation of the *FMR1* gene is critical in the pathogenesis of FXS, assessment of promoter methylation within our stem cell model was important. We found that within the pluripotent state, *FMR1* methylation was highly skewed

Fig. 2 *FMR1* expression (iso 1 and iso 10/10b) in fibroblasts. *FMR1* mRNA quantification was expressed as a fold change, relative to *GAPDH* and transcription amongst the control subjects (pooled). **a, b** A p value of < 0.05 was considered significant. Using a Bonferroni correction for multiple comparisons, a p value of < 0.0125 was

significant (**c, d**). *FMR1* total (iso 1) was underexpressed in the PM carriers (pooled), relative to controls (**a**). This pattern was preserved when examining the FB from individual PM carriers with FXPOI, as indicated by the yellow star ($p < 0.0125$) (**c**). Expression of iso 10/10b mirrored that of iso 1 (**b, d**)

(sporadic hypermethylation of one allele with demethylation of the other, regardless of *FMR1* CGG repeat size) amongst clones derived from the same input FB. To date, no prior study has looked at iPSC derived from female PM or full mutation carriers (who inherently carry two copies of the *FMR1* gene). Prior reports using stem cell models derived from male full mutation carriers have demonstrated corroborative findings [31, 32]. As it was expected that by reprogramming FXS FB into pluripotent stem cells, the *FMR1* promoter region would exhibit global demethylation, these methylation patterns could be explained by the fact that the iPSCs may not have all the characteristics of early pluripotency, but that they represent a later stage of human development [33, 34]. Alternatively, currently undefined and unique molecular mechanisms specific to the *FMR1* promoter gene within the pluripotent state could allow for seemingly complete allelic hyper and hypomethylation that may not persist beyond differentiation into primordial germ cells. This subsequent assessment will be of utmost importance, as upon differentiation from iPSC to neuronal progenitor cells, de Esch et al. observed that neural progenitor cells derived from the FXS and unmethylated full mutation iPSCs lacked *FMR1* expression and maintained a fully methylated *FMR1* promoter [34].

Whereas hypermethylation of the promoter region of the *FMR1* gene results in transcriptional silencing, leading to FMRP deficiency and intellectual disability amongst full mutation carriers [35], PM carriers may exhibit a CGG-dependent, nonlinear, elevation in expression levels of *FMR1* mRNA (2- to 8-fold in lymphocytes) and lowered FMRP levels [36], which are currently thought to play a role in the pathogenesis of the spectrum of disorders these patients develop over their lifespan. Unique to FXPOI patients, the risk of ovarian dysfunction appears to depend on repeat size; however, the relationship is not linear (peak risk falls within mid-range of repeats (60–100), and within that range, female PM carriers exhibit a modest increase in *FMR1* expression (1.5- to 4-fold) [13, 36, 37]. Whether or not this subtle change in mRNA is detrimental to ovarian function and oocyte quality and number is unclear. As it is our ultimate goal to derive oocytes from our stem cell cohort, to address the aforementioned uncertainties, we assessed both transcriptional and translational activity of the *FMR1* gene in the pluripotent state. Furthermore, given the work by Pretto et al., revealing a disproportionate (4- to 6-fold) increase of a particular isoform (10 and 10b), relative to *FMR1* total (iso 1), amongst affected PM

Fig. 3 *FMRI* total mRNA expression amongst iPSC clones. *FMRI* mRNA total (iso 1) quantification was expressed as a fold change, relative to *GAPDH* and transcription amongst the control subjects

(pooled). Where expression of each clone (A1–3) was compared to that of the parent fibroblast (FB). Using a Bonferroni correction for multiple comparisons, a *p* value of < 0.017 is significant (indicated by yellow star)

carriers (FXTAS) [38], we examined relative expression of total *FMRI* mRNA (*iso1*) and that of *iso10/10b*. When grossly comparing relative transcription of *FMRI* (*iso1* and *10/10b*) amongst the iPSCs to the parent FB, we found maintenance of FB transcriptional activity within the pluripotent stem cells (*p* = 0.76 and 0.05, respectively). More closely examining clonal behavior to that of their input FB, we found that

Table 3 FMRP production across two cell lineages

FMRP pg/μg lysate							
	<i>n</i>	Mean	Median	St dv	Min	Max	<i>p</i>
Control FB	3	27.70	28.80	2.17	25.20	29.10	
Control iPSC	9	23.89	24.40	4.56	14.70	30.20	0.20
PM FB	4	28.65	28.00	5.94	22.20	36.40	0.81
Patient 1 iPSC	3	17.77	20.30	6.11	10.80	22.20	0.09
Patient 2 iPSC	3	23.60	22.70	3.34	20.80	27.30	0.92
Patient 3 iPSC	3	25.87	26.10	5.55	20.20	31.30	0.55
Patient 4 iPSC	3	22.77	24.40	4.38	17.80	26.10	0.72

Comparison of FMRP production within FB and iPSCs amongst PM carriers and control subjects. The clones (A1–A3) were pooled for statistical analysis. Comparison of FMRP concentration (median) between FB derived from controls and PM carriers, and patient-specific iPSCs to controls exhibit statistically similar quantities of production

expression of *FMRI* (*iso 1*) was overall slightly reduced, with the exception of the clones derived from patient 4. This reduction in expression, however, had a modest effect size (as the variation was < 1 Cq unit) and did not correspond with allelic hypermethylation. Whether or not the subtle variance in *FMRI* transcription amongst the iPSC derived from PM carriers, relative to controls and input FB, is physiologic or clinically meaningful remains to be answered. Furthermore, whether the mathematical variation we see in analyzing our replicates with small variance translates to a significant biological impact is unlikely and warrants additional investigation. Additionally, changes in gene expression levels according to PM carrier or non-carrier/normal status may simply be cell lineage specific. Thus, our findings at present will serve as a reference point upon in vitro germ cell differentiation. Additionally, upon future development of a new assay that accurately segregates expression from one *FMRI* allele against another, using allele-specific anchor sequences, we may be able to expand on a more complete molecular and biological picture of *FMRI* behavior in iPSC models.

Because mid-range PMs are associated with peak risk of POI (as high as 24% [6, 7]), yet only mild elevations in *FMRI* mRNA (and in our case slightly repressed), assessment of FMRP production amongst our iPSCs was important.

Table 4 Comparison of phenotype with *FMRI* gene and protein expression profiles for premutation carriers

	Patient 1	Patient 2	Patient 3	Patient 4	Control ^a
POI	Yes	Yes	Yes	Yes	No
<i>FMRI</i> allele 1	30	32	32	23	28 (SD = 4)
<i>FMRI</i> allele 2	82	78	83	77	33 (SD = 4)
% Meth allele 2 ^a	37	23	22	14	56 (SD = 10)
% Meth allele 2 ^b	24	46	44	63	36 (SD = 7)
<i>FMRI</i> (iso 1) rel expres ^c	0.62 (SD = 0.04)	0.56 (SD = 0.05)	0.75 (SD = 0.02)	0.52 (SD = 0.08)	1.00 (SD = 0.14)
<i>FMRI</i> (iso 10/10b) rel expres ^c	0.62 (SD = 0.10)	0.60 (SD = 0.04)	0.75 (SD = 0.02)	0.52 (SD = 0.02)	1.00 (SD = 0.13)
FMRP (pd/ μ g)	29.3	26.7	36.4	22.2	27.7 (SD = 2.17)

Allele 1(a) is the smaller of the two alleles, whereas allele 2(b) is the larger. *FMRI* mRNA quantification was expressed as a fold change, relative to *GAPDH* and transcription amongst the control subjects (pooled)(c)

^a All values obtained from patient-derived fibroblast cultures, where the data from the controls was pooled for comparison

Carriers of large PM alleles are thought to exhibit a reduction in FMRP secondary to impedance of the translation initiation complex by the long RNA CGG repeat track; however, most studies have found FMRP at grossly normal levels in carriers. Kenneson et al. evaluated FMRP levels amongst male PM carriers (105–190 CGG repeats) and two males with intermediate alleles (48 and 55 CGG repeats), reporting significantly diminished FMRP levels in PM (22–40%) and intermediate range (17%) carriers as compared to controls (19–35 CGG repeats) [39]. More recently, with an improved, highly sensitive immunoassay, LaFauci et al. examined FMRP levels from dried blood spots of both men and women with normal CGG repeat number, PM, nonmosaic full mutations, and mosaic full mutations [27]. Amongst the female PM carriers, they reported an 11.5% reduction in FMRP, as compared to controls [27]. We employed that same immunoassay to assess FMRP levels within our iPSC lines and found that as compared to the input FB, the iPSCs produced similar levels of FMRP. Furthermore, we found no significant difference in FMRP levels amongst the iPSC derived from the control subjects and PM. Although relative concentrations of FMRP could be similar, FMRP functionality was not established in this study. Given evidence of high concentrations of FMRP within ovarian follicles, perhaps alterations in FMRP function that have not been clearly explained are contributing to the POI phenotype.

In addition to examining any potential effect of pluripotency on the *FMRI* gene, this study sought to correlate clinical findings with *FMRI* mRNA and FMRP levels within the patient-derived FB. Our data, from a small cohort of female PM carriers with and without POI, does not entirely corroborate the hypothesis that elevations of *FMRI* mRNA directly correlate with phenotype (POI). Transcription of *FMRI* (both iso 1 and iso 10/10b) was modestly reduced amongst all PM carriers, relative to controls. It is important to note, however, that absolute difference between carriers and controls (examining both isoforms) was < 1 Cq unit. This difference falls well within the range of broader variability reported in other studies that have

compared mRNA expression amongst PM and controls. Although *FMRI* mRNA is generally thought to be increased in PM, most compelling amongst PM carriers with > 80 CGG repeats, the relationship is imperfect and *FMRI* expression data are known to be variable [13, 38, 40]. Exploring the possibility that unique isoforms may behave more virulently, we did not appreciate an increase in isoform 10/10b relative to that of *FMRI* total (iso 1) mRNA amongst the FB derived from our PM carrier patients. Whether or not another untested isoform is disproportionately expressed within our PM carriers remains of interest. Although FXPOI and FXTAS patients share the *FMRI* PM, it is important to note the inherent differences in the two phenotypes (CGG-related peak risk, age of onset), which may be secondary to subtle, yet-to-be discovered molecular or cell-specific pathologies, such as mitochondrial dysfunction reported by Hukema et al. [22]. Certainly, additional studies are needed to evaluate whether or not accumulation of particular isoforms predisposes PM carriers to one pathology or another.

Our FMRP quantification corroborates the similarities in gene expression between cases and controls, as our analysis (Tables 3 and 4) failed to show an appreciable difference between the two groups for the important functional readout at the level of *FMRI* protein. Given that most female PM carriers exhibit normal to only slightly decreased levels of FMRP, assessment of FMRP function, in addition to quantification, would be of interest, particularly when comparing the FXPOI patients from PM with normal ovarian function.

In summary, this is the first study to create a model system, using iPSCs derived from FB of female *FMRI* PM carriers (with and without POI), designed to explore molecular mechanisms contributing to primary ovarian insufficiency and *FMRI* CGG repeat instability. In addition to the novelty of this project, this study is strengthened by the utilization of iPSCs derived from patients with well-defined clinical phenotypes, thus providing a link between reproductive manifestations related to the *FMRI* PM and in vitro phenotypic expression patterns. In order to explore such expression patterns, we

utilized highly sensitive testing platforms to establish a comprehensive, baseline characterization of key functional attributes of *FMRI*, from DNA to protein. With the exception of alterations in *FMRI* methylation seen within our iPSCs, transcription and translation were preserved to similar levels found within the input FB. Thus, we believe that our iPSCs are ready to be differentiated into germ cells, which will provide novel and important information. Although our small, case control study prevents statistical power to draw definitive conclusions regarding correlation between molecular findings and patient phenotype, we did not find an increase in *FMRI* mRNA amongst our PM carriers (regardless of phenotype). Limited to four cases and three controls, the comparative mRNA levels may not be quantitatively analogous with the trend reported in much larger cohorts. However, our findings do fall well within the range of broader variability reported in other studies that have compared mRNA expression amongst PM carriers and controls. As the peak risk of POI (up to 24% of PM carriers [6, 7]) is within CGG repeat range that is not typically associated with a substantial increase in *FMRI* mRNA, additional etiologies need to be explored. The development of FXPOI is likely multifaceted and reliant upon unidentified mechanisms. Utilizing our model system, uniquely designed from *FMRI* PM carriers with and without POI, we hope to explore molecular variabilities that may contribute to the development (or protection against) of FXPOI. Our ability to define and understand such mechanisms may likely translate to an improved understanding of not only FXPOI, but also that of idiopathic POI.

Acknowledgments The authors would like to thank Dr. Renee Reijo Pera for the initial conception of the project, and technical support in stem cell culture, and Drs. LaFauci, Dobkin and Brown, at Institute for Basic Research in Developmental Disabilities, for their FMRP analysis using the Luminex immunoassay. This work was funded by U54HD068158.

Author contributions S.G. and V.B. conceived the project. S.G., V.S., and G.L. designed the experiments. S.G., G.W., and G.L. performed the experiments. S.G., V.B., G.L., and V.S. helped with inputs and interpretation of data. S.G. wrote the paper, and all authors read and approved the final manuscript.

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